Novel Object Recognition in *Octopus maya*.

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Abstract

The Novel Object Recognition task (NOR) is widely used for the study of memory in vertebrates and has been proposed as a solid candidate for evaluating memory in different taxonomic groups, allowing similar and comparable evaluations between them. Although, in cephalopods, several research reports could indicate that they recognize objects in their environment, so far, it has not been evaluated as an experimental paradigm, which allows evaluating different phases of memory. In this study, we show that *Octopus maya* can differentiate between a new object and a known one, with high accuracy. We observed that to achieve object recognition, octopuses use vision and touch exploration in new objects, while familiar objects only need to be explored in a tactile way. To our knowledge, this is the first time showing an invertebrate performing the NOR task, in a similar way to how it is performed in vertebrates. These results raise the opportunity to use a simple, fast and widely used task in other taxonomic groups, to assess memory in octopuses.

Introduction

Cephalopods are a group of invertebrates that show a wide variety of behaviors; these behaviors manifest cognitive capacities such as learning (Fiorito and Scotto, 1992; Tomita and Aoki, 2014; Bublitz et al. 2017), emotions (Kuba et al. 2006), puzzle-solving (Richter et al. 2016) and individual recognition (Tricarico et al. 2011). These abilities are shared by different taxonomic groups, for example, insects (Simons and Tibbetts 2019), crustaceans, arachnids, and vertebrates (Roth 2013). It is important to note that the nervous system of every one of these groups differs from each other, for more than 550 million years of evolutionary history; therefore, having these cognitive capacities in each one of them probably is an evolutionary convergence. This convergence has attracted the attention of various research groups in order to understand how cephalopods achieve these capacities, mainly memory. Especially, one of the strategies to study this is by comparing cephalopods and vertebrates (Shigeno et al. 2018); but beyond this, the study of memory in cephalopods may help us understand the conditions and constraints required to achieve different types of memory in a nervous system completely different from that of vertebrates, understanding memory as a cognitive capacity that arises from neural networks, regardless of its evolutionary history.

To achieve this, it is necessary to establish a task that different taxa can perform, and that may be evaluated similarly in each one of them. A good candidate for this is the novel object recognition (NOR) task (Blaser and Heyser 2015). This task is based on the innate behavior of animals when encountering a new object in a familiar environment; the response can be exploration or aversion. The NOR task consists of indirectly evaluate the memory through discrimination based on familiarization that is, when observing a differential behavior between the presentation of a new object and a familiar one, we could infer that there is a memory of the last one. It also has several important characteristics to evaluate in the memory processes, for example, it does not require conditioning, has high ecological validity, is performed quickly, it can be divided into phases (acquisition, consolidation and retrieval), as well as temporality (long and short-term memory) and finally it has been observed that it is a task shared by all groups of vertebrates

and maybe some invertebrates (Blaser and Heyser 2015). This task has been widely studied in vertebrates, for example in fish such as sharks and zebrafish (Toms and Echeverria, 2014; Fuss et al. 2014); birds such as pigeons (Spetch et al. 2006), crows (Stöwe et al. 2006) and mammals such as Rhesus monkeys (Rajalingham et al. 2015), mice (Leger et al. 2013) and rats (Mathiasen and DiCamillo 2010). Conversely, NOR has been poorly studied in invertebrates, some similar tests have been carried out in cuttlefishes and insects, like object discrimination and context discrimination (Billard et al. 2020; Solvi et al. 2020; Kelman et al. 2008), but not in the same way as the NOR task in vertebrates, so there is still much research to be done in the field.

Although in octopuses the possibility of remembering objects or places has been anecdotally and experimentally described (Mather 1991), so far experimental tasks like the NOR task has not been standardized. That is why in this study our aim was to standardize this task in *Octopus maya* (Voss and Solis 1966), to obtain a method for the complete evaluation of memory in this octopus species and at the same time offer a standardized task that allows applying NOR task in other cephalopod species.

Materials And Methods

2.1. Ethical statement

Although there is no bioethics protocol for cephalopod laboratory experimentation in Mexico, all the experiments and handling were carried out under the approval of the bioethics committee of the Faculty of Psychology, UNAM and in accordance with the directive 2010/63/EU (European parliament), considering the recommendations by Fiorito et al. 2015.

2.2. Subjects

The specimens were obtained from the Applied Ecophysiology Laboratory of the UMDI, SISAL. *O. maya* specimens of three different ages were used; a group of five specimens four weeks post-hatching ("babies"), another group of five specimens four months old ("young") and, the last group of five specimens of unknown age but weighing more than 800 g, for which were specimens in the reproductive stage ("adults"). The groups of "babies" and "young" specimens were raised and maintained in laboratory conditions until the end of the experiments, while the group "adults" was made up of captured specimens. The animals were kept in 120 L tanks, with artificial seawater (35 salinity) and suitable conditions, according to Rosas et al. 2014.

2.3. Novel Object Recognition Task

This task was adapted from other animal models according to the octopus behavior (Lupetow 2017; Ennaceur 2018; Rossato et al. 2019), with modifications. Three phases were executed; the habituation

phase, in which the animals were put in a new enclosure and allowed to get used to the new environment for 24 hours, in this case, the animal was considered to be habituated if it was placed in a position called "rest in a den", subsequently, it was rewarded with food. After 24 hours the familiarization phase began (second phase). In this phase two equidistant objects were presented, which should be approximately the size of the mantle of the specimen used in the test, to avoid aversion or indifference on the part of individuals. These two objects were identical and were presented to "young" and "adult" for 30 minutes, and 5 minutes for the "babies" group. The difference in the duration of the test was due to the behaviors shown by the individuals at different ages as demonstrated in the results. Once this time had elapsed, the objects were removed and allowed 24 hours to pass for the last phase, that we called "the test". During the test, two objects were presented; a familiar one, taught the day before and a new one, different from the familiar object. The duration of this phase was the same as in familiarization. During the familiarization and test phases the total exploration time, the visual and the tactile exploration of the objects were quantified. Visual exploration was defined as starting locomotor activity in the direction of the object or with at least one of the eyes directed towards the object. Whereas tactile exploration was defined as touching the object with at least one of the arms. (Fig. 1).

Insert Figure 1 here.

2.4. Statistical analysis

The data was evaluated using the discrimination index ((Novel-Familiar)/Total exploration) and the non-parametric Mann-Withney U test with the software STATISTICA 10 to compare the exploration time of the familiar object with the novel one, considering $p < 0.05$ as statistically significant.

Results

3.1 When juveniles or adults *O. maya* differentially explore a known object and a new one.

Both the young group and the adult group showed similar behavior with two types of object exploration; visual and tactile guided exploration. The visual exploration consisted of indirectly approaching one of the objects, with climbs or jumps and with at least one of the eyes directed towards the object. These movements can continue until they get as close to the object as possible but without touching it. The tactile guided exploration phase is when the specimen adheres with two or more arms to the object, considering that these objects were not attached to the tank walls, hence, the specimens could lift or push them. After the objects were explored, the animals returned to their dens in a resting position. In the young and adult groups, it was observed that the exploration times were similar between both objects during familiarization and, although the time dedicated to each type of exploration could vary, the five specimens presented an approach and manipulation of both objects (Fig. 2A). After 24 hours, the evaluation phase was carried out and, it was observed that the adult specimens explored the familiar object for less time, compared to the novel object ($p < 0.05$) (Fig. 2B), while the young explored the novel object in a tactile way for a significantly less time ($p < 0.02$), but they showed the same visual exploration time ($p > 0.05$). Interestingly, it was observed that when exploring the familiar object there was no

manipulation of it, that is when the object is known, only visual exploration is used for its identification, but not tactile exploration (Fig. 2C).

Insert Figure 2 here.

For the "young" group, a counterbalance was made with the objects, for the familiarization, it was presented on one occasion two opaque white objects and on another occasion two translucent red objects. This was done to avoid a phenomenon described in the literature of preference for some colors in other octopus species (Tomita and Aoki 2014). Regarding, it was observed that although there is a greater exploration of the familiar object when it is translucent red, this difference is not statistically significant (p > 0.05); also, the differential response is maintained with the tactile exploration between the novel object and the familiar object (Fig. 2D).

3.2 At 4 weeks *O. maya* does not behave differentially with respect to the novel object and the familiar one.

When conducting the test in the "babies" group, an immediate "attack" behavior towards the objects was observed, regardless of the size of the objects, the specimens pounced on them immediately as they entered the water. For this reason, there was no division regarding the exploration phases, only the time in tactile exploration and the total exploration time (the time spent outside the den or in an activity other than rest) were evaluated. In these specimens, it was observed that although there is an increase in the time they spend in the tactile exploration of the novel object, compared to the familiar object, this difference did not occur in all individuals and is not statistically significant (p > 0.05) (Fig. 2E). On the other hand, the total time spent exploring was the same regardless of the test phase (familiarization or evaluation) and they spent significantly less time exploring than the adult group (Fig. 3).

Insert Figure 3 here.

Discussion

The novel object recognition task is a simple and short-term task that has been proposed as ideal for evaluating and comparing the memory of different groups of animals. However, it must be adapted to the characteristics that each group presents, considering the behaviors to be evaluated, times, characteristics of the objects and phases. In the present study, it was observed that young and adult specimens of *O. maya* showed novel object recognition. This recognition is observed as a decrease in the total exploration time of the familiar object. The exploration can be at least visual and tactile, the main difference being that when the object becomes familiar, it is practically not explored in a tactile way. The results suggest that the familiar object can be recognized visually, without the need to explore it with another sense, while when the object is unfamiliar, the two most prominent senses of the species are used to explore it. Unlike what was observed in the "young" and "adults", most of the "babies" did not show adequate recognition of a novel object. Also, we suspect that at the babies stage it could be more important the tactile, rather than visual exploration because of the behavior of individuals to pounce on the objects with all eight arms, in a

similar way to the attack behavior reported for other species of octopuses, for example, *O. vulgaris* (Shomrat et al. 2008; Zarrella et al. 2015). These results could suggest that *O. maya* goes through a maturation stage, during which more complex responses are acquired allowing better discrimination of the objects to be explored.

As far as we know, this is the first time this task has been applied to a species of octopus and similar adaptations have only been made in a few invertebrates such as cuttlefish (Kelman et al. 2008; Billard 2020) and bumblebees (Solvi et al. 2020). However, these were not strictly NOR tasks, but adaptations or another type of discrimination, while in this case the octopuses were tested with an identical task as it is performed in vertebrates, thus facilitating the comparison between both at a behavioral and cognitive level. On the contrary, the effect that "familiarity of context and objects" has on learning in some species of octopus has been widely described (Fiorito et al. 1998; Borrelli et al. 2020), however to our knowledge, it had not been standardized in a test that could be compared with other groups and measurable as a memory test, until now.

Also, *O. maya* presents an innate exploration behavior to novel objects, in a similar way to what happens in murine models (Ennaceur 2018), but different from what happens in other vertebrates such as Danios, who have an aversion to novel objects or neophobia (Fuss et al. 2014). Although similar, this exploration occurs much more slowly than it does in the rat and mouse models. Another relevant difference between rats and octopuses is the presence of a den during the test, which is used in order to avoid the stress of the specimens and achieve correct habituation. This den provokes a particular behavior in the animals in which the latency to explore increases and once the exploration of the objects is completed, they return to rest in their den. Though this is an important difference between the task in the murine model and octopuses, it does not influence the ability to evaluate the memory of the familiar object. Regarding the exploration time, the adult specimens total exploration time decreased significantly during the test phase. Even though in the murine model this would normally represent a problem of lack of motivation or motor skills during the test phase, in *O. maya* it can be explained taking into account that the specimens returned to their resting position in the den, once the exploration of the objects was completed. During the test phase, the familiar object was not always explored, since the total exploration time was reduced because in this lapse the animals almost only invested the time to explore the novel object.

In vertebrates, NOR has been associated with the activity of the hippocampus, insular cortex, perirhinal cortex, and medial prefrontal cortex (Tanimizu et al. 2017; Rossato et al. 2019; Cinalli et al. 2020;). Although, no invertebrate has such structures, including octopuses like *O. maya*, this and probably other octopuses species show an evolutive convergence with vertebrates in the ability to remember a familiar object. Some structures in the central brain of octopuses have been related to memory tasks and similarity has been sought between these and the structures of the vertebrate brain, particularly its vertical lobe has been compared with the hippocampus of vertebrates (Shomrat et al. 2015; Shigeno and Ragsdale 2015; Shigeno et al. 2018). While this comparison helps to try to understand what happens in the octopus brain, from our knowledge in other more studied groups, it might be important to consider a complete system, rather than a similarity for each structure.

To integrate the knowledge of how the octopus brain is working, studies were made, dividing the supraesophageal region of the octopus brain into two systems; one that includes the vertical lobe and the superior frontal lobe (VL-SF) responsible for visual memory tasks, and another that involves the buccal lobe and the inferior frontal lobe (Bu-IF), responsible for somatosensory memory tasks (Wells & Young 1975). This division is useful when evaluating tasks such as visual discrimination (Sutherland 1962; Tomita et al. 2014) or fear to condition (Shomrat et al. 2008), however, in the results of this work it is clear that in the NOR task, *O. maya* uses visual and tactile exploration. So possibly this task involves both systems, the VL-SF and the Bu-IF. This is supported by the histological observations presented elsewhere (Jung et al. 2018) as there is a neuronal connection between the superior frontal lobe and the inferior frontal lobe directly and through the sub frontal lobe.

This poses a physical substrate for the connectivity of the possible systems that are required for *O. maya* to remember the familiar object. Giving rise to propose a series of experiments that allows corroborating whether these systems participate and what would be the role that the different structures of the brain would be playing during the NOR. It would be interesting to see if visual exploration is essential for NOR in *O. maya*, since as seen in this study, once the octopus becomes familiar with the objects, it does almost not require tactile exploration to recognize it.

The present study gives evidence of evolutionary convergence between vertebrates and *O. maya* that allows the recognition of novel objects in the environment. Essentially, this task can be evaluated almost identically between both groups and opens the opportunity to compare the physiological processes that underlie it, such as neuronal plasticity, protein and RNA synthesis, the participation of different transcription factors and systems of brain structures that compose it, between others.

Declarations

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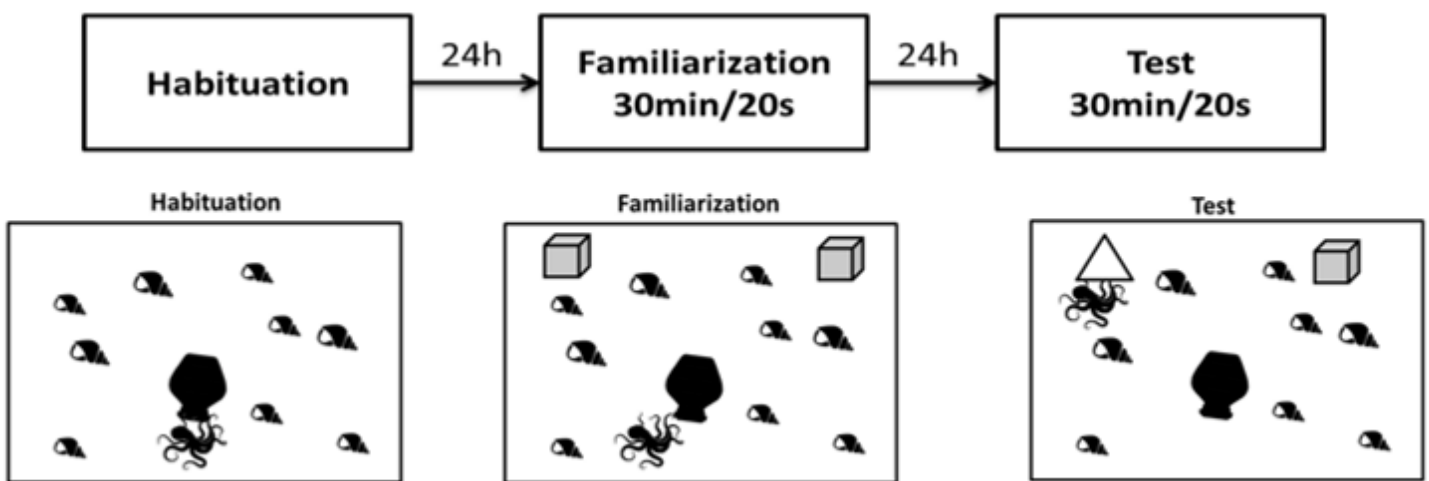
Figures

Figure 1

Experimental diagram of the novel object recognition task for *O. maya.* During familiarization, the stage of memory acquisition occurs, while in the test phase the evocation of memory is observed.
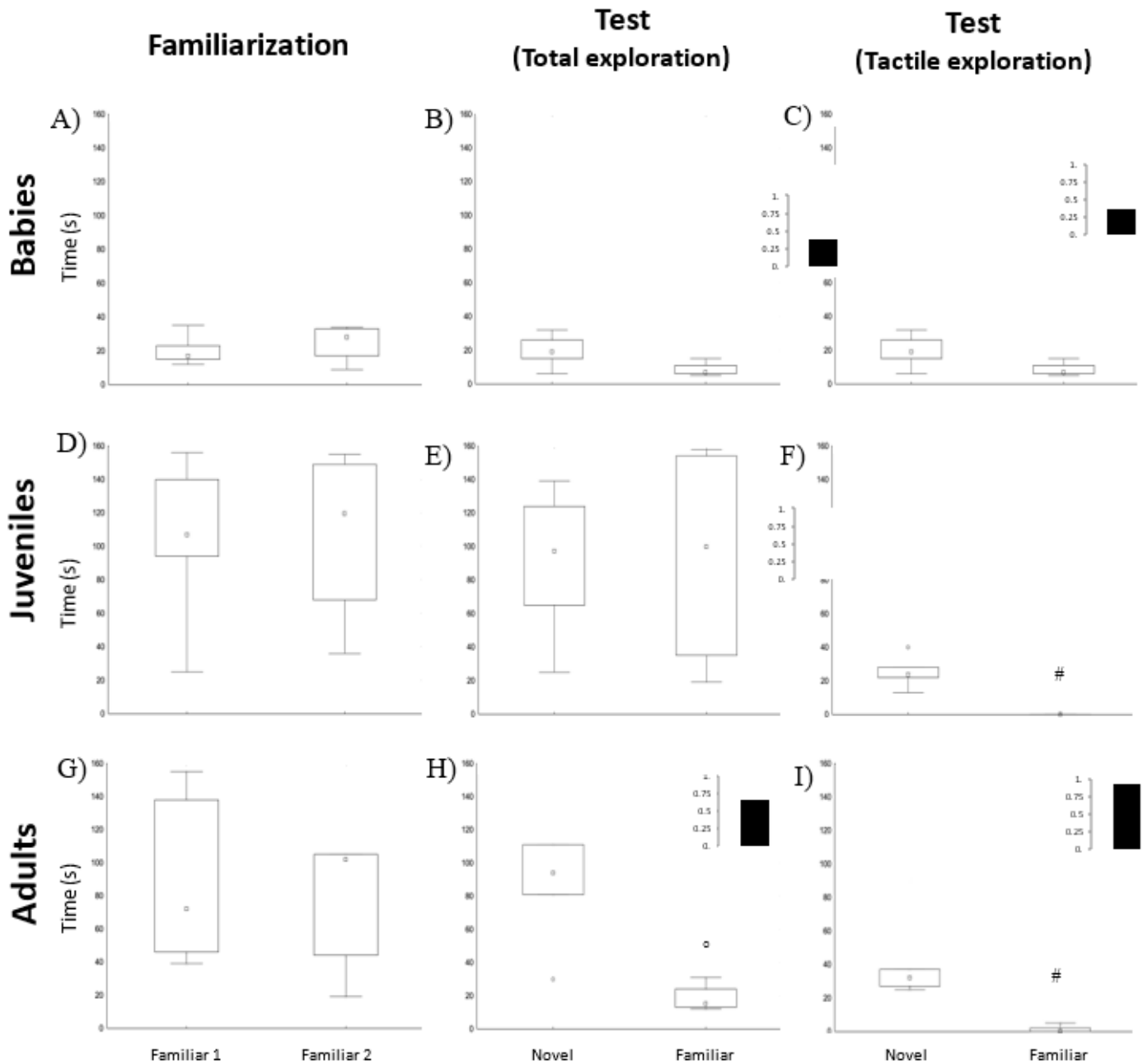
Figure 2

Results of the NOR test in babies (A, B, C), juveniles (D, E, F) and adults (G, H, I) of *O. maya*. During the familiarization phase (A, D and G) there are no differences between the total exploration time of both objects, although it is observed that babies explored for less than 60 seconds, while the young and adult groups explored for more than 100 seconds. In the total exploration during the test phase (B, E, H), only adults (H) show a significant difference (° $p < 0.05$). If only tactile exploration is considered, both young and adult groups showed a differential behavior between the novel and familiar object (# $p < 0.02$). The black bar graphs show the discrimination index ((Exploration of the novel object - Exploration of the familiar object) / Total exploration) the f graph has a mistake in the index
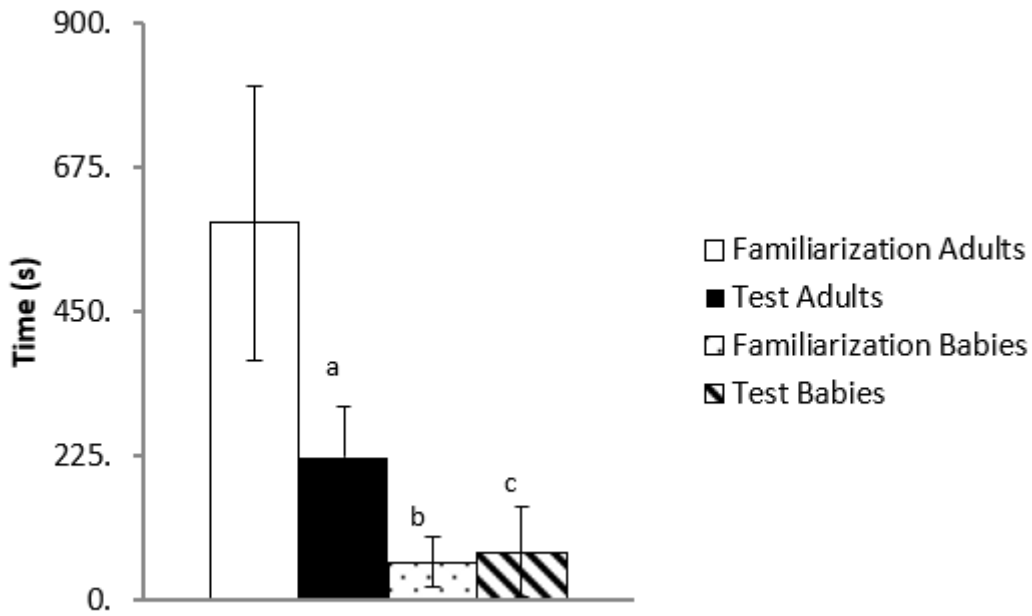
Figure 3

Total exploration time during the NOR test in "adult" and "baby" groups. The total exploration time in adults was shorter during the test phase (p <0.05) (a). The babies group explored significantly less time than the adults (p <0.02) (b), however, they did not show differences between the familiarization and test phases (c).